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Using the Yeast Two-Hybrid System to Determine the Function of Parkin E3 Ubiquitin Ligase

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USING THE YEAST TWO-HYBRID SYSTEM TO DETERMINE THE FUNCTION OF PARKIN E3 UBIQUITIN LIGASE

by

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ABSTRACT

Parkin is a cytosolic E3 ubiquitin ligase that is recruited to the mitochondria during cellular stress and has been suggested to be involved in a variety of biological processes such as mitophagy. The recruitment of Parkin (PARK2) to the mitochondria is dependent upon the kinase activity and the accumulation of PINK1 on damaged mitochondria. Mutations in either PINK1 or Parkin genes disrupt this protective pathway and lead to the accumulation of damaged mitochondria. From a clinical standpoint, mutations in the PARK2 gene have been associated with the progression and onset of autosomal recessive juvenile parkinsonism. Without the presence of a quality control system such as that of the PINK1/Parkin pathway, the accumulation of damaged mitochondria could lead to increased levels of oxidative stress, a decrease in ATP, and the progression towards cellular death. However, many of the details regarding the mechanism of Parkin-mediated ubiquitination and its involvement in mitophagy are not fully established.

The intent of this thesis is to further explore the function of Parkin by utilizing the yeast-two hybrid system to identify novel Parkin interactors/substrates. A HeLa (cervical cell carcinoma) cDNA library was screened using $Parkin_{124-465}$ as the "bait" protein. From this screening, six positive Parkin interactors were isolated and characterized. Using this approach it is possible to gain a better understanding of the function of Parkin in regulating cellular processes such as mitophagy.

DEDICATIONS

For my family, Bic Vu, Tho Nguyen, and David Nguyen, thank you all for your unwavering encouragement throughout my academic endeavors and for motivating me to persevere for the goals I aspire to achieve.

For Daniel Washburn, thank you for visiting me in the lab during your spare time, for listening to my desperate pleas for my yeast to turn blue, and for your constant support since the start of this project.

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CHAPTER ONE: INTRODUCTION

Clinical Significance

Parkinson's disease (PD) is considered to be the second most common neurodegenerative disorder and is characterized by four primary clinical symptoms: tremor, bradykinesia, rigidity, and postural instability [1]. Reduced dopamine neurotransmission due to the damage of dopaminergic neurons in the substantia nigra and the presence of Lewy body inclusions in nerve cells are pathological hallmarks of this incurable disease; however, the molecular mechanism of how these neurons become impaired is unclear [1]. While the majority of diagnosed PD cases are classified as sporadic and idiopathic, genetic studies of rare familial forms of PD have provided information about the significance of protein degradation pathways and mitochondrial dysfunction in the development of PD [1, 2]. Mutations in specific genes encoding for alpha-synuclein and leucine-rich-repeat kinase 2 (LRRK2) have been connected to autosomal dominant forms of PD, while mutations in PTEN induced putative kinase 1 (PINK1) and Parkin genes have been associated with autosomal recessive forms of PD [1-3].

Aggregation of damaged mitochondria has been implicated in a variety of age-related disorders including PD and Alzheimer's disease [1]. Damaged mitochondria are selectively removed by the process of mitophagy, in order to prevent the activation of cellular death (apoptosis) [4, 5]. Studies have revealed that a physical interaction between PINK1 and Parkin serves as a mitochondrial quality control system that promotes the selective degradation of defective mitochondria in mammalian cells [3, 6]. PINK1 encodes a serine/threonine kinase that contains a mitochondrial targeting sequence that allows for its localization to the mitochondria

[1, 3, 6]. In healthy mitochondrion, PINK1 is imported into the inner mitochondrial membrane where it is rapidly cleaved and degraded by various proteases [3, 4]. The stabilization and build-up of PINK1 on the outer mitochondrial membrane is considered as a mechanism for detecting mitochondrial dysfunction [4, 5].

Parkin is a cytosolic E3 ubiquitin ligase that translocates to the mitochondria during cellular stress and is speculated to be involved in a variety of biological processes, including mitophagy, cell survival pathways, and vesicle trafficking [6, 7]. The recruitment of Parkin to the mitochondria is dependent upon the kinase activity and the accumulation of PINK1 on damaged mitochondria [3, 4, 6]. Upon translocation to the mitochondria, the ubiquitin activity of Parkin increases leading to activation of mitophagy [7]. Mutations in either PINK1 or Parkin genes disrupt this protective pathway and lead to accumulation of damaged mitochondria [1, 4-6]. In relation to PD, neurons in the substantia nigra region of the brain are particularly vulnerable to the effects of mitochondrial dysfunction [4]. Without the presence of a quality control system such as that of the PINK1/Parkin pathway, the accumulation of damaged mitochondria could lead to increased levels of oxidative stress, a decrease in ATP, and the progression towards cellular death [6]. However, many of the details regarding the mechanism of Parkin-mediated ubiquitination and its involvement in mitophagy are not fully established [7, 8]. This project will utilize a yeast-two hybrid system to identify novel E2 conjugating enzymes and other potential proteins that interact with Parkin and define the mechanism of its normal function.

The presence of numerous mutations and rearrangements in the PARK2 gene has been linked as the cause to early onset form of PD known as autosomal recessive juvenile

parkinsonism (AR-JP) [1, 2]. It has been suggested that individuals with mutations in the Parkin gene generally display symptoms in their early thirties, though the age of onset can vary widely [9]. Furthermore, this implies that because there is an earlier onset of Parkinson's disease in individuals with Parkin mutations, patients over the age of thirty can attribute the onset of the disease to causes other than Parkin mutations [9]. In regards to the symptoms experienced by those diagnosed with AR-JP, they are more likely to experience dystonia and hyperreflexia, but in comparison to those with idiopathic Parkinson's disease, such individuals tend to have a better response to levodopa but have a higher risk of experiencing dyskinesia during treatment [9]. As for the specific location of where the mutations occur within the Parkin gene, the use of PCR-based techniques has proposed that the exon deletions, exon multiplications, truncating mutations, and missense mutations are predominately clustered within the RING-IBR-RING finger motif; however, the effects of the various mutations on the Parkin's normal have not been fully elucidated [9].

Structural Information

Analyzing the structural components of Parkin can reveal new information regarding its potential function and activation. The PARK2 gene encodes a protein, referred to as Parkin, consisting of 465 amino acids and contains an N-terminal ubiquitin-like (Ubl) domain and a C-terminal RING2 (Really Interesting New Gene) domain [2, 3, 7, 10]**.** In between the N-terminus and the C-terminus lie two additional RING finger regions– RING0 and RING1 – and an additional in-between RING (IBR) finger that separates RING1 and RING2 (Figure 2) [2]. The RING-type zinc finger domain is characterized by a specific sequence of cysteines and histidines folded around two zinc cations, and this configuration provides stability and plays a key role in mediating protein-protein interactions [10].

The surface of the Ubl domain appears to be bound to the RING1 region via hydrophobic interaction and has the capability of binding to ubiquitin-interacting motifs and SH3 domains upon dissociation from RING1 [10]. Compared to other proteins containing RING finger regions, the RING0 domain is an atypical structure that is unique to Parkin [10]. Specifically, it is involved in coordinating two zinc atoms at each end of the domain with a hairpin structure [10]. The RING1 domain appears to be the only domain that resembles the common $Cys_3HisCys_4$ sequence and zinc coordination pattern ("cross-brace" motif) found in other RING fingers [10]. Furthermore, studies have revealed that this specific domain contains an E2 binding region, thus highlighting its involvement in Parkin's E3 ubiquitin ligase activity [8, 10]. The IBR region participates in maintaining conformational flexibility, and it has been demonstrated that the missense mutation Gly328Glu located in the hinge region between RING1 and IBR can affect Parkin's positioning and stability [10]. The RING2 domain displays a similar sequential

topology to that of the IBR region and contains two zinc-coordinating residues that interact with a hydrophobic groove in RING0 [10]. Each domain within the structure of Parkin has a specific function, thus mutations occurring within these regions can result in a variety of effects that can inhibit Parkin's activity.

Overview of Ubiquitination Process

Similar to the function of other RING finger proteins, Parkin acts as an E3 ubiquitin ligase in the ubiquitin-proteasome system (UPS) [2, 8, 10]. UPS is identified as the principle mechanism for proteolysis in eukaryotic cells; it is an ATP-dependent process that involves the conjugation of ubiquitin, a highly conserved 76-amino acid polypeptide, to proteins that are destined for degradation by the proteasome [2]. Ubiquitin can be attached through the carboxy group of a glycine residue found in its C-terminus (Gly76) to a specific lysine in the target substrate [5]. UPS occurs through successive reactions involving three classes of enzymes: ubiquitin–activating (E1), ubiquitin–conjugating (E2), and ubiquitin–ligase (E3) enzymes (Figure 1) [8]. The hydrolysis of one ATP molecule drives the formation of a thioester bond between a cysteine in the E1's active site and the carboxy-terminus of ubiquitin [8]. The ubiquitin moiety, which is now activated upon interaction with the E1, binds to an active site cysteine residue on the E2 enzyme resulting in the formation of a new thioester bond [8, 11]. The E2 enzyme is involved in conjugating the active ubiquitin to a specific substrate bound to an E3 ligase [11].

Depending upon the different classes of E3 enzymes, the ubiquitin can be conjugated directly or indirectly to the E3-bound substrate [11]**.** The HECT (homologous to the E2-AP COOH terminus) ligases utilize an indirect process in which the ubiquitin is transferred from the E2 to an active site cysteine residue on the E3 thus creating an ubiquitin-thioester intermediate [11]**.** Following this formation, the ubiquitin is then transferred to the ligase-bound substrate [11]. On the contrary, RING-type ligases catalyze the direct transfer of ubiquitin to the target substrate thus facilitating as a scaffold protein to bring the substrate and the E2 closer together

[7, 11]. In essence, the E3 confers substrate specificity and catalyzes the covalent attachment of ubiquitin to the ε-amino group of an internal lysine residue in the substrate [8, 12]. The conjugation of ubiquitin to the substrate can occur once (monoubiquitination) or multiple times thereby creating a polyubiquitin chain [8, 10]**.** Ubiquitin elongation involves the linkage of the C-terminal glycine residue (Gly76) of the new ubiquitin monomer to a specific lysine residue - Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63 - found in the previously ligated ubiquitin [2, 11]. The degradation of the ubiquitin-tagged substrate by the 26*S* proteasome complex is contingent upon the length and the type of the ubiquitin-chain formed [8]. Of the components involved in the UPS, the E3 ligases play a pivotal role in determining the fate of the targeted protein since these enzymes perform the following tasks: provide the specificity of which proteins should be targeted with ubiquitin, determine the length of the ubiquitin chain, and decide at which positions the poly-ubiquitin molecules should be linked [8, 11].

As for the number of identified ubiquitin enzymes, there are two E1s, more than 30 E2s, and over 1000 E3s known to exist in eukaryotes [11]. In regards to its E3 classification, Parkin is specifically identified as a RING-in-between-RING (RBR) ligase [10]**.** It has been revealed that this group uses both RING and HECT-like mechanisms to perform the ubiquitin transfer [12]. Additionally, a key characteristic that distinguishes the RBR ligases from other types of E3 ligases is the use of an auto-inhibitory mechanism that modulates ubiquitination activity [12]. The precise mechanism and understanding of Parkin's role in the ubiquitination of mitochondrial substrates has not been well established [2, 3, 7, 8, 10, 12]**.** Very little is known about RBRs role in regulating substrate recognition and more specifically, the interaction of Parkin with its E2 enzymes is poorly understood [8, 12]. Recent investigations on Parkin's activation and

enzymatic function at mitochondria have revealed preliminary information about its interaction with its E2 enzymes [2, 7]. It is believed that the E2 enzymes that interact with Parkin play a significant role as direct or indirect regulators of Parkin's involvement in mitophagy [7]. In addition, the appearance of distinct ubiquitin-linkages with specific lysines (Lys48, Lys63, Lys27) suggests further support for the involvement of E2 enzymes in Parkin-mediated mitophagy [7]. Thus, by identifying new E2 partners of Parkin and other potential substrates and clarifying their functions, it is possible to further determine the mechanism of Parkin's regulation and function.

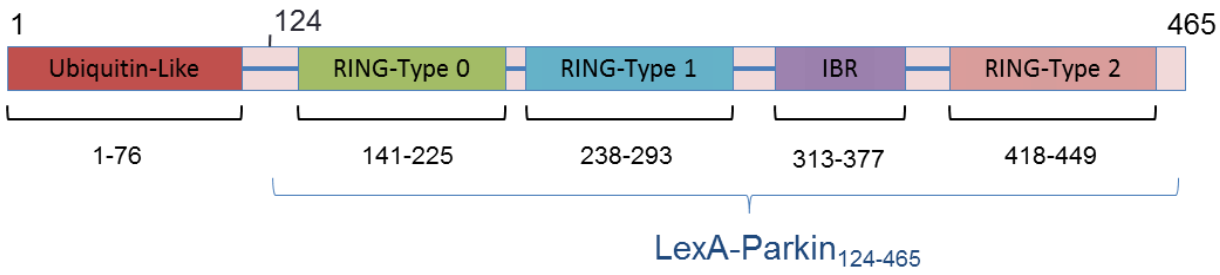

Figure 1: Schematic diagram of E3 ubiquitin-protein ligase Parkin indicating the various domains present throughout its sequence.

The ubiquitin-like domain is located in the N-terminus region and is speculated to be involved in binding ubiquitin-interacting motifs. Unique to parkin, the RING0 domain coordinates two zinc atoms with a hairpin. The RING1 domain, which contains an E2 binding site, displays the common cross-brace zinc coordination that is typical of other RING domains. The IBR region is involved in maintaining Parkin's conformational flexibility. The RING2 domain has a similar topology to that of the IBR domain.

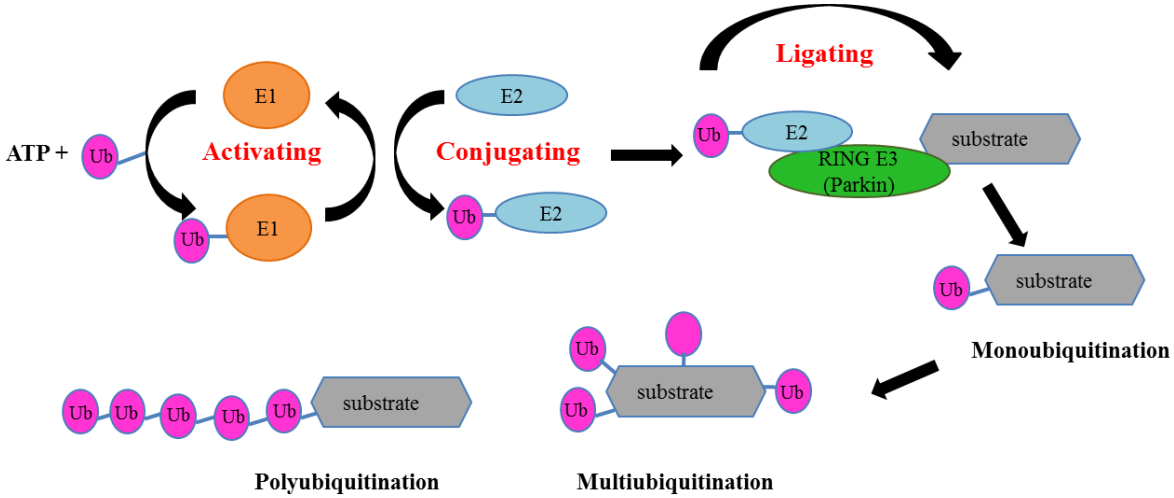

Figure 2: Schematic overview of the ubiquitination system.

The ubiquitination of a target protein is performed by three different enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-ligating enzyme (E3). The activation of ubiquitin is an ATP-dependent process that occurs through the formation of a thioester bond with E1. When the ubiquitin is attached to E2, it can be directly or indirectly transferred to the substrate. The E3 enzyme facilitates bringing the E2-ubiquitin complex closer to the substrate. The fate of the substrate is dependent upon the number and the type of ubiquitin chain.

CHAPTER TWO: MATERIALS AND METHODS

Yeast Two-Hybrid System

The yeast two-hybrid system is a valuable technique that can be utilized to identify interactors of a certain protein of interest [13]. The nature of these interactors can define the function of the protein and provide information on the mechanism involved. Furthermore, this method can be used to identify the domains or residues that are involved in the interaction between the two proteins [13]. In summary, the "bait" refers to the protein of interest, while the potential protein interactors are identified as the "prey". The basis behind the yeast two-hybrid system is to generate two hybrid proteins: the bait fused to a LexA DNA-binding domain and the prey fused to an activation domain [13]. When these two proteins are separated, neither is able to initiate transcription of the reporter gene *lacZ*; however, the interaction between these two hybrid proteins activates the transcription of *lac*Z [13]. The benefit of using this reporter gene is that *lacZ* encodes for the β-galactosidase enzyme which produces an intense blue color to the yeast product when the medium contains the substrate X-gal [13]. After performing various tests to eliminate false negatives, the presence of blue colonies on the appropriate medium allows for the identification of clones that provide a positive protein-protein interaction with the protein of interest [13].

Previous Use of Yeast Two-Hybrid System

In Zervos lab, the yeast two-hybrid screen was previously used successfully to identify specific E2 conjugating enzymes of Mulan, an E3 ubiquitin ligase that is embedded in the outer mitochondrial membrane [14]. It contains a RING finger domain facing the cytoplasm and an additional large domain in the intermembrane space [14]. Though the mechanism of its function is currently unknown, Mulan is an important factor in a variety of cellular processes, such as cell growth, cell death, and autophagy/mitophagy [14]. The initial yeast two-hybrid screen revealed four E2 conjugating enzymes that were capable of forming complexes with Mulan. After determining the E2 partners of Mulan, an additional, modified yeast-two hybrid screen was performed to identify possible interactors for each Mulan-E2 heterodimer complex [14]. From this screening, several distinct interactors for each Mulan-E2 complex were isolated and analyzed, thus revealing critical information regarding Mulan's involvement in mitophagy [14]. This study serves as an example of how the identification of protein-protein interactions can reveal valuable information about the normal function of a specific protein.

Polymerase Chain Reaction (PCR)

The forward and reverse primers, which flank the cDNA sequence of Parkin (coding for amino acids 124-465), contain an EcoRI and BamHI restriction site (respectively).

Forward: 5'-GCGGAATTCCACACTGACAGCAGGAAGGACTC-3'

Reverse: 5'-CGCGGATCCCTACACGTCGAACCAGTGGTC-3'

In order to amplify the target DNA sequence, a PCR reaction was performed using these primers. The reaction mixture contained a total volume of 50 µl and included the following components: 20 ng of template DNA, 10 nM in 1 µl of each primer, 5 µl of 10X PCR Buffer + Mg2+, 1.5 units in 1 µl of TAQ Polymerase, 10 µM of dNTPs, and 40.8 µl of sterile H2O. This mixture was made in duplicate and placed in a thermal cycler to perform the reaction. The PCR program consisted of an initial denaturation step at 95℃ for two minutes and 25 cycles of the following steps: denaturation at 95℃ for 30 seconds, annealing at 60℃ (dependent upon primers) for 30 seconds, and elongation at 72℃ for 70 seconds. The final step, known as the incubation period to ensure that any single-stranded DNA is fully extended without any gaps, lasted for 3 minutes at 72℃. After performing this procedure, the duplicate mixtures were combined and precipitated to isolate the PCR DNA product.

Restrictive Enzyme Digestion

In preparation for DNA ligation, a restriction enzyme digestion was performed. The reaction mixture of each digestion contained a total volume of 100 µl and was incubated at 37℃ for two hours. Plasmid DNA was first digested with BamHI restriction enzyme using the following components: 3 µl of pGilda DNA (2.25 µg/µl), 10 µl of BamHI 10X buffer, 1 µl of 100X BSA, 1.5 µl of BamHI, and 84.5 µl of sterile H_2O. In a separate reaction, the PCR product was initially digested with BamHI. This reaction mixture contained 25 µl of the PCR product, 10 µl of 10X BamHI buffer, 1 µl of 100X BSA, 62.5 µl of sterile H_2O, and 1.5 µl of BamHI. The product from the BamHI digestion was then precipitated in preparation for the second restriction enzyme digestion with EcoRI. The pellet from the precipitation was combined with 10 µl of 10X EcoRI buffer, 1 µl of 100X BSA, 1.5 µl of EcoRI, and 87.5 µl of sterile H_2O. After the PCR product digestion with BamHI, the product was then precipitated, and the pellet was combined with 10 µl of 10X EcoRI buffer, 1 µl of 100X BSA, and 87.5 µl of sterile H_2O, and 1.5 µl of EcoRI. DNA gel electrophoresis was performed to estimate the DNA concentration of pGilda and to determine whether the plasmid digestion was complete.

DNA Ligation

Following restrictive enzyme digestion, the DNA insert was ready for ligation into pGilda. This specific ligation reaction mixture consisted of the following: 1.5 µl of 10X Ligation Buffer, 1.5 µl of 10 mM ATP, 1 µl of DNA ligase, 8 µl of insert, and 4µl of vector. In general, a 3:1 ratio of insert DNA to vector is used to determine the appropriate amount. The reaction mixture was first incubated for 5 minutes at room temperature and then transferred to a 70℃ water bath for 15 minutes to deactivate DNA ligase. The final volume of the samples was brought up to 50 µl and an additional 500 µl of N-butanol was added in order to precipitate the DNA. After centrifuging the samples for 10 minutes (13,000 RPM) at 4℃, the supernatant was discarded, and the sample was dried in a speed vacuum for 10-12 minutes. The pellet was then resuspended in 4-6 µl of sterile H_2O.

Bacterial Transformation

For bacterial transformation, 4-6 µl of the DNA ligation product was added into a 100 µl aliquot of competent bacterial cells (DH5α), mixed gently, and then transferred to an electroporation cuvette that was cooled to 4℃. The cuvette was placed in the Gene Pulser (Biorad) to deliver a single exponential decay pulse of 2.5V. Following the shock, 700 µl of room temperature LB medium without any antibiotics was added immediately to the cuvette. The mixture was then transferred to a 2 ml microcentrifuge tube and incubated at 37℃ for one hour. Used as the negative control, the plasmid without the insert was also transformed following the same protocol. After the incubation period, different volumes of the mixture (150 µl and 250 µl) were plated on LB agar plates containing ampicillin (the selectable marker present in pGilda) and incubated overnight at 37℃. The following day, 30 single bacterial colonies were picked from the plates, and each colony was grown overnight in 1.5 ml of LB media containing 50 µg/ml Ampicillin.

Quick Isolation of Plasmid DNA from Bacteria (Miniprep)

The DNA boiling method (Miniprep) was used to quickly isolate plasmid DNA from the bacterial colonies. The suspensions grown overnight were transferred to 1.5 ml Eppendorf tubes and centrifuged for 2 minutes at a speed of 13,000 RPM. After aspirating the supernatant, the pellet was resuspended in 300 µl of a STET/lysozyme mixture (8% Sucrose, 5% 100X Triton, 50 mM Tris-HCL (pH 8), 50 mM of EDTA, and 600 µl Lysozyme (10 mg/ml)). The samples were briefly vortexed, placed in a boiling water bath for 1 minute, and centrifuged for 10 minutes (13,000 RPM). The pellet, which contained cellular debris, was removed with a toothpick and discarded. In each tube, 200 µl of Isopropanol was added, and the samples were mixed thoroughly before centrifugation for 10 minutes (13,000 RPM). After removing the supernatant, each pellet was washed with 200 µl of 70% ice-cold ethanol and centrifuged for an additional 5 minutes. Following centrifugation, the ethanol was removed, and the DNA pellet was dried in a speed vacuum for 10 minutes. 50 µl of TE was added to each tube to resuspend the DNA. 3 µl from each tube was then checked with DNA gel electrophoresis to determine which samples could be used in the double digestion with both restriction enzymes.

DNA Digestion

To ensure that the insert DNA was properly ligated into pGilda, ten Miniprep samples were selected for a double digestion with EcoRI and BamHI. Each of the reaction mixtures contained the following: 2 µl of 10X EcoRI Buffer, 0.2 µl of 10X BSA, 0.3 µl of EcoRI, 0.3 µl of BamHI, 4 µl of Miniprep DNA, and 13.2 µl of sterile H_2O. After incubating the mixtures for 4 hours at 37℃, the samples were checked with DNA gel electrophoresis to analyze which samples contained the insert. The undigested samples of the selected Minipreps were run side-by-side with the digested samples. If two bands were present for the digested sample, this signified that this specific clone was positive and contained the insert. Eight of the ten Miniprep samples were positive, and of the positives, six were selected to transform the yeast.

CHAPTER THREE: RESULTS

Yeast Two-Hybrid Screening

For the yeast two-hybrid screening, the EGY48 strain with the LexA-β galactosidase reporter construct (PSH 18-34) was used as the host to screen for protein interaction. The yeast was then transformed using 1 µl of the LexA-Parkin$_{124-465}$ plasmid DNA from the positive Miniprep samples, plated on U^-H^- Glucose plates, and incubated for 2-3 days at 30℃. A single yeast colony was grown overnight in U^-H^- Glucose media and induced for four hours the following day. To determine whether the target protein was expressed, a Western blot was performed using LexA antibodies. The upper band of the Western blot represents the protein expression of LexA-Parkin$_{124-465}$, which is estimated to be around 57 kDa (Figure 3).

Before continuing with the screening, a color test was performed to ensure that the bait cannot self-activate, and a growth test was conducted to verify that the bait cannot activate the endogenous Leu^- gene required for growth on selective media. For the color test, one of the clones from the yeast transformation was grown overnight in U^-H^- glucose media and the next day, a yeast transformation was performed using the pJG4-5 empty vector. Various volumes were plated on $U^-H^-W^-$ Glucose media and incubated for 2-3 days. Single colonies from the transformation were then streaked on a $U^-H^-W^-$Galactose/Raffinose X-Gal agar plate to detect the presence of blue colonies (Figure 4). The results from this test reveal that LexA-Parkin$_{124-465}$ + pJG4-5 (empty) did not turn blue thus signifying that it is not self-activating. Additionally, the growth test revealed that LexA-Parkin$_{124-465}$ + pJG4-5 (empty) did not grow on the $U^-H^-W^-L^-$ Galactose/Raffinose plate (Figure 5).

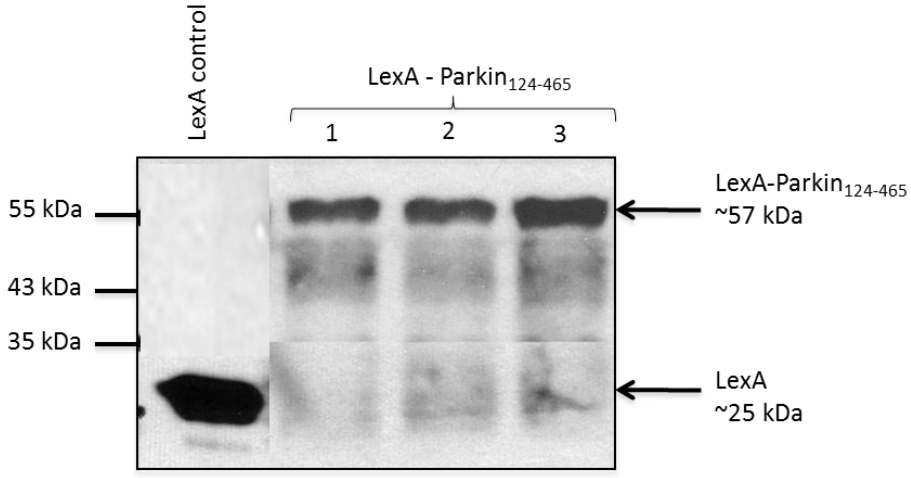

Figure 3: Western blot to verify the expression and stability of LexA-Parkin$_{124-465}$ in yeast.

Six yeast colonies transfected with LexA- Parkin$_{124-465}$ and one colony with the pGilda vector alone were grown in U$^-$H$^-$ galactose media; yeast were lysed and proteins separated by SDS-PAGE. Expression of the recombinant proteins was monitored using LexA specific antibodies.

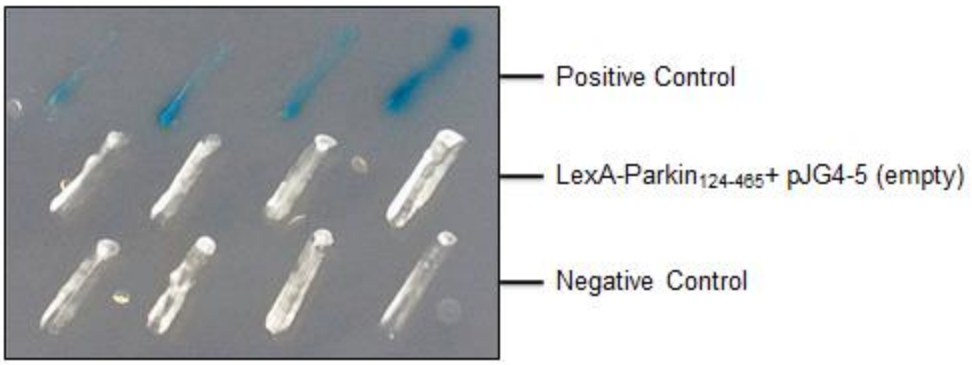

Figure 4: Color test to verify lack of self-activation of the LexA-Parkin$_{124-465}$ bait in yeast.

Yeast colonies transfected with LexA-Parkin$_{124-465}$ were streaked onto a $U^-H^-W^-$ Galactose/Raffinose/X-Gal plate. Blue color developing after overnight incubation indicates activation of the reporter β-galactosidase gene. LexA-Parkin$_{124-465}$ did not self-activate the reporter gene and as such can be used in the yeast two-hybrid screening.

Figure 5: Growth test to further verify that the LexA-Parkin$_{124-465}$ bait cannot activate endogenous Leu$^-$ gene.

Different dilutions of yeast transfected with LexA-Parkin$_{124-465}$ as well as a positive and negative control were spotted onto U$^-$H$^-$W$^-$L$^-$Galactose/Raffinose plates. After 3 days, the growth of yeast expressing LexA-Parkin$_{124-465}$ was monitored by comparing it to the positive and negative controls.

High Efficiency Yeast Transformation

To ensure the highest efficiency and yield of transformants to be used in the library screening, a high efficiency yeast transformation was performed. The key difference between a high efficiency transformation versus a standard yeast transformation is that the yeast-bait culture must be grown to a specific OD_{600} of 0.9 before being utilized. A single yeast colony of EGY48 pSH18-34 LexA-Parkin$_{124-465}$, was picked and grown overnight at 30 °C with shaking in 50 ml of $U^{-}H^{-}$ Glucose drop out medium. The next day the culture was diluted to 200 ml with the same medium and grown to an OD_{600} nm of 0.924. Cells were harvested by centrifugation at 3800 rpm for 5 minutes. The supernatant was discarded; cells were washed with 20 ml of sterile water and centrifuged for an additional 5 minutes. After another wash cycle, cells were resuspended in 20 ml LA (0.1M LiOAc in TE), spun again for 5 minutes. Lastly cells were resuspended in 5ml of LA. 100 µl of competent yeast was aliquoted in 2 Eppendorf tubes and the following components were added: 10 µg DNA (HeLa cDNA library in pJG4-5), 100 µg of denatured salmon sperm DNA (carrier DNA) and 600 µl of freshly prepared 40% PEG solution. The mixtures were resuspended until homogeneous. The transformation reaction was then incubated at 30°C for 30 minutes and then heat shocked at 42°C for 15 minutes. Following brief centrifugation of the samples to form a pellet, the supernatant was aspirated. The pellet was resuspended in 500 µl of sterile water. 300 µl of resuspended yeast was then plated on 22.5 x 22.5 cm $U^{-}H^{-}W^{-}$ Glucose selective plates and incubated at 30°C for 2-3 days. In total, ten big plates were created and used for the aliquoting of the library.

Screening from Big $U^-H^-W^-L^-$ Galactose/Raffinose Plates

After plating on the big $U^-H^-W^-L^-$ Galactose/Raffinose plates for growth selection, large colonies were selected and streaked onto smaller $U^-H^-W^-L^-$ Galactose/Raffinose plates with the use of toothpicks. Following an incubation period at 30°C for 2-3 days, the colonies from these plates were then re-streaked onto $U^-H^-W^-$ Glucose to be used as a master plate. From this plate, the colonies were streaked onto $U^-H^-W^-$ Glucose/X-Gal and $U^-H^-W^-$ Galactose/Raffinose/X-Gal plates for color selection. A positive interaction between the bait and the prey was denoted with the presence of a blue colony on $U^-H^-W^-$Galactose/Raffinose/X-Gal and a corresponding white colony on $U^-H^-W^-$ Glucose/X-Gal. The presence of blue colonies on both $U^-H^-W^-$Galactose/Raffinose/X-Gal and $U^-H^-W^-$ Glucose/X-Gal signified false positives. Figure 6 depicts the blue colonies on $U^-H^-W^-$ Galactose/Raffinose/X-Gal; however, not all of these colonies were true positives.

After determining the colonies that turned blue only on $U^-H^-W^-$Galactose/Raffinose/X-Gal, the corresponding colonies were picked from the $U^-H^-W^-$ Glucose master plate and inoculated into W^- glucose medium for selection of the pJG4-5 HeLa library plasmid. The culture was grown overnight at 30°C with shaking in preparation for the release of the plasmid from the yeast.

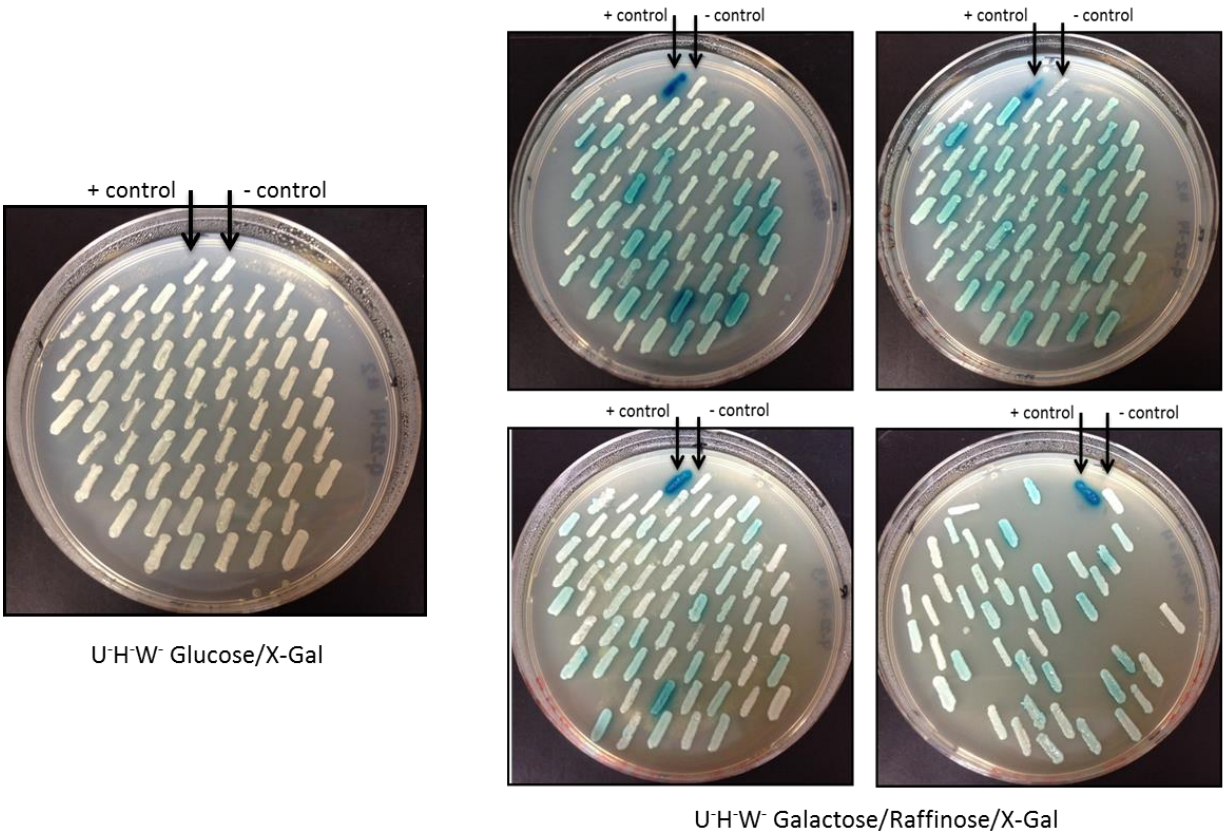

Figure 6: HeLa cDNA library screening.

A positive interaction between LexA-Parkin$_{124\text{-}465}$ and the prey proteins leads to blue colonies on U$^-$H$^-$W$^-$Galactose/Raffinose/X-Gal whereas white colonies indicate that there is no interaction. Additionally, blue colonies on U$^-$H$^-$W$^-$Glucose/X-Gal correspond to false positives.

Release of Plasmid from Yeast

The yeast culture grown in W^- Glucose medium was transferred to a screw cap microcentrifuge tube and was centrifuged for 1 min at 13000 rpm. After aspirating the supernatant, the formed pellet was resuspended in 200 µl of yeast lysis solution (2% TritonX-100, 1% SDS, 100mM NaCl, 10mM Tris pH8.0, 1 mM EDTA). Approximately 0.3 g of glass beads (Sigma) was added to assist in the lysis along with 200 µl of Phenol-chloroform-isamyl alcohol (25:24:1). To homogenous the mixture, each culture was vigorously vortexed for 2 minutes using a Mini-Beadbeater machine. Following this procedure, the cultures were centrifuged for 5 minutes to separate each sample into three distinct, visible layers. The top layer contained the desired DNA; the middle layer contained protein, while the bottom layer contained debris. Without penetrating the middle layer and contaminating the DNA, the top layer was extracted from each sample and added to a new microcentrifuge tube for precipitation.

Bacterial Transformation of DNA Plasmids from the Yeast into KC8 Cells

Following precipitation of the extracted DNA plasmids, the samples were transformed into bacterial cells (KC8 strain) utilizing the same method as discussed in the previous chapter for bacterial transformation. After electroporation and incubation for an hour, 250 µl of each sample was plated on WM9 ampicillin (AMP) plates and incubated overnight at 37℃. In preparation for isolation of the plasmid DNA, a single colony from each plate was grown overnight in 2 ml of LB media containing 50 µg/ml Ampicillin.

Determination of True Positive Interactors of LexA-Parkin$_{124-465}$

The DNA boiling method, as described in the previous chapter, was utilized to isolate the plasmid DNA. The key aspect of the bacterial transformation into KC8 cells and isolation of the DNA plasmid via Miniprep is to determine the specific prey plasmid that is interacting with the bait. Whereas yeast can carry multiple types of plasmids, bacteria are more selective in that each bacterial cell can only contain a single type plasmid. Therefore, the bacterial transformation with the KC8 cells and the isolation of the plasmid DNA is a crucial step in order to narrow down the true positive interactors with the bait and the specificity of the prey plasmid.

The isolated plasmid DNA was transformed back into the same yeast strain containing only the bait (EGY48 pSH18-34 LexA-Parkin$_{124-465}$) and each sample was plated on $U^{-}H^{-}W^{-}$ Glucose. The colonies that grew from these plates were then streaked onto U-H-W- Glucose/X-Gal and U-H-W- Galactose/Raffinose/X-Gal for color selection. This time around, a blue colony on U-H-W- Galactose/Raffinose/X-Gal and a corresponding white colony on U-H-W- Glucose/X-Gal signified a true positive interaction (Figure 7).

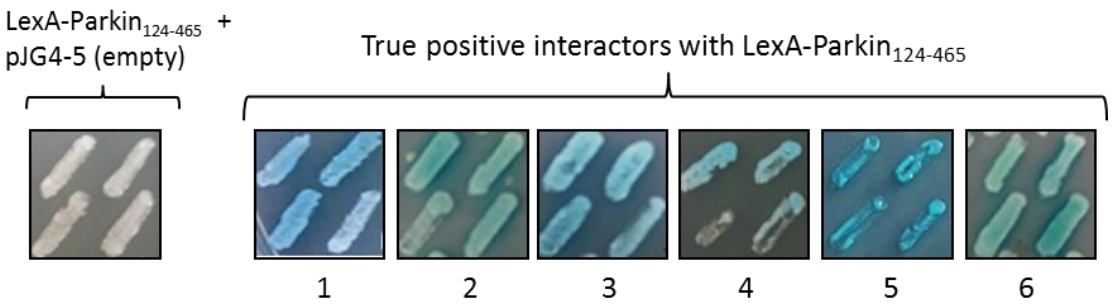

Figure 7: True positive interactors with LexA-Parkin$_{124-465}$.

To determine whether the blue colonies from the library screening were true positives, the corresponding blue colonies were grown in W^- media in preparation for the release of the plasmid from the yeast. The plasmids were then extracted from the yeast, transformed into KC8 bacterial cells, and plated onto WM9 ampicillin plates. The plasmid DNA, which was isolated via the DNA boiling method, was transformed back into the yeast with the bait protein and streaked onto $U^-H^-W^-$Glucose/X-Gal and $U^-H^-W^-$Galactose/X-Gal. Colonies that were blue on $U^-H^-W^-$Galactose/X-Gal but white on $U^-H^-W^-$Glucose/X-Gal signified a true positive interaction with the bait protein.

Clean DNA QIAprep Isolation of DNA from Bacteria

In preparation for sequencing, the plasmids were isolated using the QIAprep Spin Miniprep Kit, and the protocol was provided by the manufacture (QIAgen). Individual bacterial colonies were grown in 2 ml of LB media and Ampicillin and incubated overnight at 37°C. The following day, these suspensions were mixed and transferred to microcentrifuge tubes, which were then centrifuged (11,000 RPM) for 2 minutes. The supernatant was aspirated, and the pellet was resuspended in 200 µl of Resuspension Buffer P1 containing RNase A (100 µg/ml). 200 µl of Lysis Buffer P2 was then added and to ensure complete cell lysis, the tubes were then gently inverted 5-6 times until the solution appeared homogenous and transparent. With the addition of 300 µl of Neutralization Buffer N3, the lysate was neutralized and adjusted to the high-salt binding conditions. The sample was immediately inverted for an additional 5-6 times until a white cloudy precipitate formed. The suspension was then centrifuged (13,000 RPM) for an additional 10 minutes. Using a pipette, the supernatant was transferred to a QIAprep spin column that is resting on top of a 2 ml collection tube. The column was centrifuged for 1 minute at 13,000 RPM and the flow-through was discarded. The column was washed with 500 µl of PB Buffer and centrifuged for an additional 1 minute at 13,000 RPM, and the flow-through was discarded. The column was then washed with 750 µl of PE Buffer and centrifuged for 1 minute at 13,000 RPM. The flow-through was once again discarded, and the column was centrifuges for an additional 1 minute to remove the residual wash buffer. After transferring the QIAprep spin column to a clean 1.5 ml microcentrifuge tube, 50 µl of EB buffer (10 mM Tris-HCl, pH 8.5) was added, allowed to bind for 1 minute, and the column was centrifuged for 2 minutes at 13,000 RPM to collect the DNA.

Sequence	LexA-Parkin$_{124-465}$ Interactors	Accession Number
1	Homo sapiens bolA family member 1 (BOLA1), transcript variant X2, mRNA	XM_006711348.1
2	Homo sapiens ATP synthase, H^+ transporting, mitocondrial F1complex, epsilonsubunit (ATP5E), mRNA	NM_006886.3
3	Homo sapiens mitochondrial ribosomal protein L19 (MRPL19), mRNA	NM_014763.3
4	Homo sapiens complement component 4 binding protein, beta (C4BPB), transcription variant 1, mRNA	NM_000716.3
5	Homo sapiens NHP2 non-histone chromosome protein 2-like 1 (S.cerevisiae) (NHP2L1), transcript variant X2, mRNA	XM_006724258.1
6	Homo sapiens lysyl-tRNA synthetase (KARS), transcript variant 1, mRNA	NM_001130089.1

Table 1: Proteins isolated as specific interactors with LexA-Parkin$_{124-465}$.

CHAPTER FOUR: DISCUSSION

Parkin (also known as PARK2) is a cytosolic E3 ubiquitin ligase that is recruited to mitochondria during times of cellular stress and participates in mitophagy [5-7]. Mutations in the PARK2 gene have been associated with the development and progression of autosomal recessive early-onset Parkinson's disease (PD) [1, 2, 9]. The complete details of Parkin's mechanism of action and involvement in mitophagy are not fully established. One of the approaches used to investigate the function of a protein is the yeast two-hybrid system. In this case, the function of a protein is defined by its specific interaction with other interactors. This approach has been recently used successfully in our laboratory to characterize the function of yet another E3 ubiquitin ligase, Mulan [5, 14]. Parkin has several distinct domains that could be involved in protein-protein interactions (Figure 2) suggesting that is has the potential to interact with multiple proteins at the same time. For this project, I have used the yeast two-hybrid system to isolate and characterize proteins that could interact with $Parkin_{124\text{-}465}$. I have excluded the 124 amino terminal amino acids from the bait because the focus of the study was to determine potential interactors with the RING domains of Parkin. In addition, it has been reported that the amino terminal of Parkin acts as an inhibitor to other protein-protein interactions [10]. For the screening, I decided to use a HeLa cDNA library that has previously been used successfully in our laboratory.

Six positive interactors were identified and characterized (Table 1). From these six interactors, based on what is known, at least one of them has the potential to be a physiological partner of the Parkin protein. This interactor is the Homo sapiens bolA family member 1, otherwise known as BolA homolog 1 (BOLA1). Although this protein has been predominately

studied in *Escherichia coli*, three homologs of the BolA protein family have been identified in human and yeast [15]. In *E. coli*, the BolA gene is upregulated under cellular stress and is involved in the regulation of cell wall proteins; however, its precise mechanism of action has yet to be clarified [15]. While sufficient experimental data analyzing the biological function of BOLA1 is lacking, it is suggested that this specific homolog has a function in regulating normal mitochondrial morphology in aerobic eukaryotes [15]. Furthermore, even though it appears to be ubiquitously expressed in humans, it is not overly-expressed in any specific tissues [15]. More studies are needed to verify whether this interactor binds to Parkin in mammalian cells and under what conditions.

Another interesting interactors identified in my screen of Parkin was the lysyl-tRNA synthetase which is encoded by the *KARS* gene [16]. This specific enzyme is responsible for the charging of $tRNA^{Lys}$ molecules and is required in both the mitochondria and the cytoplasm in order for protein translation to occur [16, 17]. In regards to its clinical application, mutations in the *KARS* gene are implicated in the onset of Charcot-Marie-Tooth (CMT) disease, which is characterized by a variety of peripheral nerve disorders, such as a decrease in motor nerve conduction velocities, axonal loss, or a decrease in the amplitudes of nerve responses [17]. Additionally, evidence suggests that *KARS* mutations in the cytoplasmic enzyme are inherited as a recessive trait, and the presence of two mutant alleles can result in the most severe phenotype of this neuropathic disorder [16]. It is hypothesized that *KARS* variants disrupt the aminoacylation process through the formation of an active Lysine tRNA synthetase (LysRS) tetramer, resulting in the interference of tRNA binding activity [16]. However, the effects of impairment and dysfunction in the mitochondrial enzyme of the *KARS* gene have not been fully

analyzed, and it has yet to be determined whether LysRS has a direct connection to the regulation of mitochondrial dynamics [16].

Since our bait ($Parkin_{124-465}$) encompasses multiple protein-protein domains within its structure, the use of smaller baits can further elucidate the exact area of Parkin that is involved in each interaction. This could therefore provide information in regards to which proteins interact with a specific RING domain and how the interaction affects the function of Parkin. Additionally, to investigate whether the protein interactors from this experiment can interact with Parkin in mammalian cells, co-immunoprecipitation experiments will be carried out to characterize whether these protein complexes form in such cells. Because Parkin is a cytosolic protein that is recruited to the mitochondria during cellular stress, another interesting point to investigate would be to determine the cellular location of the protein interaction with Parkin, whether it is in mitochondria or in the cytosol. Furthermore, since Parkin is classified as an E3 ubiquitin ligase, future experiments will be performed to analyze whether Parkin ubiquitinates the interacting proteins in order to gain a better understanding of its mechanism of action in the ubiquitin pathway. From this project, I have shown that the yeast two-hybrid system can be used to study protein-protein interaction using $Parkin_{124-465}$ as the bait. Future experiments using the results from this screening will be carried out to further characterize the function of Parkin and its involvement in regulating cellular processes like mitophagy.

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